Published in final edited form as:
Gastroenterology. 2011 July ; 141(1): 176–185. doi:10.1053/j.gastro.2011.04.010.

MyD88 and Retinoic Acid Signaling Pathways Interact to Modulate Gastrointestinal Activities of Dendritic Cells

Eduardo J. Villablanca[1], **Sen Wang**[1], **Jaime De Calisto**[1,2], **Daniel C. O. Gomes**[3], **Maureen A. Kane**[4], **Joseph L. Napoli**[4], **William S. Blaner**[5], **Hiroyuki Kagechika**[6], **Rune Blomhoff**[7], **Mario Rosemblatt**[2,8], **Maria Rosa Bono**[2], **Ulrich H. von Andrian**[9], and **J. Rodrigo Mora**[1,*]

[1] Gastrointestinal Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

[2] Facultad de Ciencias, Universidad de Chile, Santiago, Chile

[3] Universidade Federal do Rio de Janeiro, Brazil

[4] University of California, Berkeley, CA, USA

[5] Columbia University, New York, NY, USA

[6] Tokyo Medical and Dental University, Tokyo, Japan

[7] Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway

[8] Fundacion Ciencia para la Vida & Universidad Andres Bello, Santiago, Chile

[9] Immune Disease Institute, Harvard Medical School, Boston, MA, USA

Abstract

BACKGROUND & AIMS—Gut-associated dendritic cells (DC) metabolize vitamin A into *all-trans* retinoic acid (RA), which is required to induce lymphocytes to localize to the gastrointestinal (GI) tract and promotes the differentiation of Foxp3+ regulatory T cells (T_{REG}) and immunoglobulin (Ig)A antibody-secreting cells (IgA-ASC). We investigated whether RA functions in a positive-feedback loop, via DC, to induce its own synthesis.

METHODS—We measured levels of retinoids in intestine tissues from mice and assessed the role of RA in activities of gut-associated DC in cell cultures and mice. We used pharmacologic antagonists to determine the signaling pathways involved in regulation of DC and used *MyD88−/−* mice to determine the contribution of Toll-like receptor (TLR) signaling in RA-mediated activities of DC.

RESULTS—The concentration of retinoids decreased in a proximal-to-distal gradient along the intestine, which correlated with the activity of gut-specific DC. Importantly, RA regulated the

*Correspondence and requests for materials should be addressed to J. Rodrigo Mora (j_rodrigo_mora@hms.harvard.edu).

Author's contributions: EJV, SW, JDC and DCG designed and performed experiments. MAK and JLN performed the retinoid measurements. HK, WSB and RB provided reagents and mice. MR, MRB and UHvA, provided valuable suggestions and advise on experiments and on the manuscript. EJV and JRM develop the conceptual framework for the study, supervised the project, designed experiments, analyzed data and wrote the paper.

Conflicts of interest: The Authors disclose no conflicts

ability of gut-associated DC to produce RA, induce T cells to localize to the GI tract, and generate T_{REG} and IgA secreting cells. RA was sufficient to induce its own production by extra-intestinal DC, *in vitro* and *in vivo*. RA-mediated regulation of DC required signaling through the mitogen-activated protein kinase signaling pathway and unexpectedly required MyD88, which has been associated with TLR, interleukin (IL)-1, and IL-18 signaling.

CONCLUSIONS—RA is necessary and sufficient to induce DC to regulate T-cell localization to the GI tract and IgA secretion. These findings indicate crosstalk between the RA receptor and MyD88-dependent TLR signaling pathways.

Keywords

Inflammation; IBD; immune response; mouse model

Lymphocyte migration is a key event during intestinal inflammation [1]. Therefore, it is critical to understand how T and B cells migrate to the intestine and how their migration patterns could be manipulated for therapeutic purposes. The gut-homing receptors integrin α4β7 and chemokine receptor CCR9 are required for T and B lymphocyte migration to the gut mucosa in the steady state and also during intestinal inflammation in mice and humans [1, 2], thus acting as molecular "zip codes" by controlling lymphocyte migration in a tissue-specific fashion.

DC from mesenteric lymph nodes (MLN-DC), Peyer's patches (PP-DC) and small intestine lamina propria (gut-associated DC), but not DC from extra-intestinal sites, induce a high expression of gut-homing receptors on lymphocytes [3–6], which is explained by their ability to metabolize vitamin A (retinol) into all-*trans* retinoic acid (RA). RA is necessary to imprint gut-homing lymphocytes [5–7], to promote differentiation of IgA-ASC [5] and to control the balance between T_{REG} and Th_{17} cells in the gut mucosa [8, 9]. Therefore, given the essential role of RA in intestinal immune homeostasis, it is important to understand how the synthesis of RA is modulated in the gut mucosa.

Here we show that RA controls RA-synthesizing capacity in DC *in vitro* and *in vivo*, hence inducing a positive feedback loop on its own synthesis and conferring DC with gut-specific imprinting properties. In addition, we found that RA-mediated DC education requires the expression of the intracellular adaptor MyD88, which is conventionally associated with TLR and IL-1/IL-18 signaling [11, 12], suggesting a novel crosstalk between RA- and MyD88-dependent pathways.

Results

DC ability to imprint gut homing correlates with retinoid levels in the gut

RA and its precursors retinol and retinyl esters were readily detected in the intestinal mucosa and their concentrations followed a gradient from proximal to distal, with the highest concentrations being found in the duodenum and jejunum (Fig. 1A). Interestingly, retinoid levels correlated with DC imprinting ability. PP-DC or lamina propria DC (LP-DC) from duodenum and jejunum induced higher levels of gut-homing receptors (α4β7 and CCR9) and $Foxp3^+$ T cells as compared to their counterparts in ileum, colon or spleen (Fig. 1B, C and Fig. S1A). Reciprocally, the skin-homing receptors E- and P-selectin ligands were more efficiently induced by ileum PP-DC/LP-DC, colon LP-DC and Spleen-DC than by duodenum or jejunum PP-DC/LP-DC (Fig. S1B), consistent with the notion that the induction of these receptors occurs as a default pathway in T cells activated without RA [7]. Consistent with their higher gut-homing imprinting capacity, PP-DC and LP-DC from duodenum and jejunum exhibited higher Raldh activity (Aldefluor staining [17]) than ileum or

colon DC (Fig. 1D), which was not explained by different proportions of $CD103^+$ DC (Fig. S1C). Of note, the expression of *Aldh1a2* mRNA (encoding Raldh2) was not significantly different among DC from different intestinal segments and their relative mRNA levels were lower than those found in MLN-DC (Fig. S1D), suggesting that *Aldh1a2* mRNA levels in DC do not always fully correlate with their Raldh activity or gut-homing imprinting capacity. Whether this dissociation between *Aldh1a2* mRNA and Raldh activity reflects local differences in Raldh protein expression or stability remains to be determined. In addition, using DR5-luciferase reporter mice, in which luciferase is controlled by a promoter with RA response elements (RARE) [13], we determined that all tested DC have the capacity to respond to RA *ex vivo* (Fig. S1E). However, consistent with their exposure to high levels of RA, DC from the proximal small intestine exhibited higher luciferase activity than distal DC (Fig. 1E).

RA is necessary in vivo for gut-associated DC education

We depleted mice of the RA precursor retinol by feeding them a vitamin A-deficient (VAD) diet, as described [5, 7]. Since vitamin A is abundantly stored in the liver it is difficult to attain complete vitamin A depletion, even when using a VAD diet for several months [14]. To address this shortcoming we used mice deficient in lecithin:retinol acyltransferase (LRAT), which cannot store retinol in the liver [14]. $LRAT^{-/-}$ mice develop normally when maintained on a vitamin A-sufficient diet, but they become vitamin A depleted after only 2–4 weeks on a VAD diet, with the additional advantage of avoiding potential unwanted effects of chronic vitamin A depletion [14]. In agreement with a critical role of RA in gut-associated DC education, PP-DC and MLN-DC from VAD mice induced lower levels of gut-homing receptors on T cells as compared to their counterparts from mice on a vitamin A-sufficient diet (Fig. 2A). In addition, MLN-DC and PP-DC from VAD mice were impaired in inducing $Foxp3^+$ T cells (Fig. 2B) and IgA-ASC (Fig. 2C), respectively. Moreover, MLN-DC from VAD mice showed a marked reduction in *Aldh1a2* mRNA expression (Fig. 2D) and an impaired Raldh activity in total and $CD103^+$ MLN-DC (Fig. 2E), which was rescued by oral RA supplementation (Fig. 2F). Thus, RA is necessary *in vivo* to educate gut-associated DC with critical gut-specific imprinting properties.

RA is sufficient to confer DC with RA synthesizing capacity in vitro and in vivo

Murine Spleen DC pre-treated with RA (RA-DC) upregulated *Aldh1a2* mRNA (Fig. 3A and Fig. S2A, B). This effect was not limited to RA, as other natural and synthetic RA receptor (RAR-RXR) agonists (Am80, 9-*cis* RA, 13-*cis* RA) also induced *Aldh1a2* in Spleen-DC. In contrast, agonists for RXR-RXR (HX630, PA024), PPARγ-RXR (Rosiglitazone), PPARβ/δ-RXR (GW0742), PXR-RXR (Lithocholic Acid), LXR-RXR (TO901317) or AHR (ITE) nuclear receptors did not induce *Aldh1a2* mRNA. RA also induced *Aldh1a2* in murine peripheral lymph node (PLN)-DC and bone marrow (BM)-derived DC (Fig S2C) as well as *ALDH1A2* mRNA in human monocyte-derived DC (Mo-DC) (Fig. S2D), suggesting that our findings could be extrapolated, at least in part, to human DC.

Consistent with their higher expression of *Aldh1a2* mRNA, RA-DC displayed higher Raldh activity as compared to untreated Spleen-DC (UT-DC) (Fig. 3B), which was also observed when treating PLN-DC and BM-DC with RA (Fig. S2E), indicating that RA-DC acquired RA-synthesizing capacity. Importantly, RA-DC induced significantly higher levels of gut-homing receptors α4β7 and CCR9 on activated T cells as compared to UT-DC, which was abrogated when T cells were activated in retinol-free media (Fig. 3C) or in the presence of the Raldh inhibitor diethylaminobenzaldehyde (DEAB) [17] (Fig. 3D), implying that the induction of gut-homing receptors by RA-DC required active RA synthesis and was not due to RA carry-over, which happens when DC are incubated with high concentrations of RA [18]. Similarly, human monocyte-derived DC (Mo-DC) pre-treated with RA induced higher levels

of gut-homing receptors on polyclonally activated human T cells than untreated Mo-DC (Fig. S2F). Adding the RAR-inhibitor LE540 during the co-culture abrogated gut-homing imprinting by RA-DC (Fig. S2G), indicating that gut-homing imprinting by RA-DC requires RA activity via RAR nuclear receptors. Consistent with their higher expression of gut-homing receptors, T cells activated with RA-DC migrated significantly more to the small intestine as compared to those activated with UT-DC (Fig. 3E). In addition, analogous to gut-associated DC [8, 9], RA-DC also promoted the induction of $Foxp3^+$ T_{REG} (Fig. 3F).

To obtain mechanistic clues on *Aldh1a2* induction by RA in DC, we incubated Spleen-DC from DR5-luciferase mice with RA in the presence or absence of the transcription inhibitor actinomycin-D or the translation inhibitor cycloheximide as described [19]. RA-treated Spleen-DC exhibited increased Raldh and luciferase activities (induction of *Aldh1a2* mRNA and a RAR-dependent reporter by RA, respectively), which were abrogated by actinomycin-D or cycloheximide treatment (Fig. S3A, B), suggesting that RA-mediated DC education to express Raldh and synthesize RA requires *de novo* transcription and protein synthesis. Moreover, actinomycin-D inhibited RA-mediated induction of *Aldh1a2* mRNA and *Rarb* mRNA (encoding RARβ, a known RA-induced gene) (Fig. S3C), indicating that their increase is mostly due to *de novo* transcription rather than enhanced mRNA stability. Interestingly, cycloheximide blocked *Aldh1a2* but not *Rarb* mRNA induction, suggesting that the increased *Aldh1a2* mRNA transcription in RA-treated Spleen-DC requires newly synthesized proteins, whereas *Rarb* appears to be directly induced by RA without requiring *de novo* protein synthesis. This finding is consistent with their distinct transcription kinetics, as *Rarb* mRNA was clearly induced as quickly as 1 hour post RA treatment, whereas *Aldh1a2* mRNA exhibited a more delayed kinetics (Fig. S3D). In sum, RA-induction of *Aldh1a2* mRNA in Spleen-DC appears to occur indirectly in a process that requires prior *de novo* protein synthesis.

Of note, although Spleen-DC acquired Raldh activity when pre-treated with RA for 24 hours, this was not observed when DC were exposed to RA for 30 min (i.e., only during the Aldefluor assay) (Fig S3E). Moreover, MLN-DC maintained the same high Raldh activity regardless of whether the mice were supplemented or not with oral RA (Fig S3F). Thus, RA does not appear to directly induce, enhance or otherwise affect Raldh enzymatic activity, suggesting that its main effect on DC education involves *de novo Aldh1a2* mRNA and Raldh protein induction.

To assess whether RA can also educate extra-intestinal DC *in vivo*, we treated mice with RA via oral gavage and then analyzed PLN-DC for their expression of *Aldh1a2* mRNA, Raldh activity and gut-homing imprinting capacity (Fig. 4A). As expected, PLN-DC from control mice expressed much lower levels of *Aldh1a2* mRNA and Raldh activity as compared to MLN-DC (Fig. 4B, C and Fig. S4A). By contrast, PLN-DC from mice treated with oral RA exhibited significantly higher levels of *Aldh1a2* mRNA and Raldh activity than PLN-DC from control mice. Moreover, PLN-DC from RA-treated mice were able to induce gut-homing receptors on activated T cells, an effect that was dependent on the presence of retinol and Raldh activity (Fig. 4D), demonstrating that it required active RA biosynthesis.

We did not detect RA in PLN from untreated animals, even using the most sensitive methods [16], whereas RA was readily detected in PLN from RA-treated animals with levels approaching those found in MLN from untreated animals (Fig. S4B). Moreover, luciferase activity in PLN cells from RA-treated DR5-luciferase mice was similar to that in MLN or PP cells from control animals (Fig. S4C).

Of note, RA supplementation was associated with increased proportions of activated $CCR9^+$ CD8 T cells in PLN and spleen upon s.c. immunization, reaching levels comparable to

control MLN (Fig. S4D). Thus, oral RA treatment increases RA concentration and RA-dependent activity in PLN to levels comparable to those in the normal MLN environment, suggesting that RA or other RAR agonists, such as 13-*cis* RA (isotretinoin) (Fig. 3A), could be used as "mucosal adjuvants" to increase gut-associated immune responses in peripheral compartments.

In summary, our data show that RA is sufficient to induce a positive feedback loop in extra-intestinal DC, conferring them with the capacity to synthesize RA and to generate gut-homing T cells *in vitro* and *in vivo*.

RA-mediated DC education requires ERK/MAPK signaling

To obtain further mechanistic insights on how RA educates DC we tested the role of canonical signaling pathways in RA-mediated DC education by adding pharmacological inhibitors concomitant with RA treatment. Blockade of p38/MAPK (SB203580), JNK/MAPK (SP600125) or NF-κB (SN50) pathways [3] did not affect RA-mediated DC education (Fig. 5A). However, inhibition of ERK/MAPK (U0126) [3] during RA treatment of DC significantly decreased their capacity to induce gut-homing receptors on T cells. Moreover, blocking ERK completely abolished *Aldh1a2* mRNA induction in RA-DC (Fig. 5B), indicating that ERK signaling is required for RA-mediated DC education. Interestingly, ERK inhibition in Spleen-DC isolated from DR5-luciferase mice abrogated RA-mediated luciferase induction (Fig. 5C), suggesting that ERK signaling might be required for general RAR-dependent effects on DC.

To assess whether ERK signaling plays a physiological role in gut-associated DC education, we treated mice with PD0325901, an orally bioavailable ERK inhibitor that has been recently used *in vivo* [15]. Consistent with a physiological role of ERK signaling in gut-associated DC education, treatment with PD0325901 significantly decreased Raldh activity in endogenous $CD103^+$ MLN-DC (Fig. 5D), without decreasing the frequency of $CD103^+$ DC in MLN (data not shown).

Thus, ERK signaling is needed for RA-mediated DC education and is also required *in vivo* for endowing gut-associated DC with RA-synthesizing capacity.

MyD88 is required for RA-mediated DC education

Given that some TLR-agonists can induce Raldh enzymes in DC in an ERK-dependent manner [20] and that RA-mediated DC education also requires ERK signaling, we explored whether TLR signals might modulate RA effects on DC [11]. Consistent with this possibility, Spleen-DC from mice lacking MyD88 (essential for most TLR signaling [11]) were impaired in their RA-mediated DC education. $MyD88^{-/-}$ RA-DC induced lower levels of gut-homing receptors on T cells as compared to wild type RA-DC (Fig. 6A) and the induction of *Aldh1a2* mRNA was completely abrogated in $MyD88^{-/-}$ RA-DC (Fig. 6B). Similarly, *Tgm2* mRNA (encoding tissue transglutaminase, a well-known RA target gene [21]) was not induced in $MyD88^{-/-}$ RA-DC, whereas it was readily induced in wild type RA-DC (Fig. 6B), suggesting that MyD88 fulfills a more general role in RA-mediated effects on DC. Of note, DC subset composition and maturation status were comparable in $MyD88^{-/-}$ and wild type Spleen-DC (data not shown).

Next, we asked whether MyD88 was required *in vivo* for RA-mediated DC education. $MyD88^{-/-}$ or wild type mice were orally supplemented with RA. Spleen-DC from RA-treated mice were used to activate naïve CD8 T cells. PLN-DC isolated from RA-treated $MyD88^{-/-}$ mice exhibited lower Raldh activity than PLN-DC from RA-treated wild type mice (Fig. 6C and Fig. S5A). Moreover, Spleen-DC from RA-treated $MyD88^{-/-}$ mice induced lower levels of α4β7 and CCR9 on T cells as compared to Spleen-DC from RA-

treated wild type mice (Fig. S5B). In addition, the expression of the RA-induced gene *Tgm2* was lower in MyD88$^{-/-}$ PP-DC as compared to wild type PP-DC, whereas *Fabp4* mRNA, a PPARγ-RXR target gene [10], was expressed at similar levels in MyD88$^{-/-}$ and wild type PP-DC (Fig. S5C). These data suggest that MyD88 expression is necessary for RAR-RXR mediated effects on DC, but is not generally required for the activity of other nuclear receptors sharing the RXR nuclear receptor partner.

Wild type and MyD88$^{-/-}$ Spleen-DC did not differ in the expression of *CrabpII* and *Fabp5* mRNA, which encode proteins that modulate RA signaling by channeling RA to either RAR-RXR or PPARβ/δ-RXR, respectively [22] (Fig. S5D).

Interestingly, RA induced *Tlr1* and *Tlr2* mRNA (Fig. S5E). Thus, RA might contribute to sensitizing DC to TLR1/2 ligands, suggesting a potential intersection point between TLR- and RA-mediated effects on DC. In addition, RA induces *Rarb* mRNA in DC (Fig. S3C, D and Fig. S5E), hence exerting a positive feedback loop in its signaling.

We found that RA-mediated education was ERK dependent (Fig. 5) and it has been shown that TLR engagement results in ERK activation in DC [20]. Moreover, RA has been shown to rapidly induce ERK phosphorylation [23], suggesting that ERK signaling might represent another potential intersection point between TLR- and RA-mediated signaling in DC. Therefore, we assessed whether RA can induce ERK phosphorylation in Spleen-DC from wild-type and MyD88$^{-/-}$ mice. Interestingly, although RA induced ERK1/2 phosphorylation in Spleen-DC, this effect was decreased on MyD88$^{-/-}$ DC (Fig. S5F, G). Of note, ERK phosphorylation was not affected in TLR4$^{-/-}$ Spleen-DC, hence excluding an effect due to LPS contamination. These results suggest that RA induces ERK1/2 phosphorylation, an effect that requires MyD88 expression in Spleen-DC.

The mechanism by which lack of MyD88 impairs RA-mediated ERK1/2 phosphorylation remains undefined, although we hypothesize that it might be related, at least in part, to decreased RA-mediated signaling. In fact, whereas wild type and MyD88$^{-/-}$ Spleen-DC did not differ in the expression of most RAR and RXR isoforms, *Rarb* mRNA (encoding RARβ) was not detected in MyD88$^{-/-}$ Spleen-DC (Fig. 6D), suggesting that lack of RARβ might explain, at least in part, the decreased RA-mediated education on MyD88$^{-/-}$ DC. In agreement with this possibility, LE540, a predominantly RARβ inhibitor [24], completely abrogated RA-mediated *Aldh1a2* induction (Fig. 6E) and RA-induced luciferase activity in Spleen-DC from DR5-luciferase mice (Fig. 6F). Thus, the mechanism by which MyD88 controls RA-mediated effects on DC might involve RARβ modulation.

TLR signals contribute to RA-mediated DC education

Since our data suggest that MyD88 is critical for RA-mediated DC education, we hypothesized that TLR-agonists contribute to DC education. Consistent with this possibility, human Mo-DC treated with RA and/or a TLR1/2-agonist (Pam_3CSK_4) exhibited higher Raldh activity as compared to untreated Mo-DC (Fig. 7A). The increased Raldh activity in Mo-DC correlated with their ability to induce Foxp3 in human CD4 T cells (Fig. 7B). Moreover, mouse Spleen-DC or human Mo-DC treated with $\alpha4\beta7^+$ and $CCR9^+$ T cells as compared to DC RA plus Pam_3CSK_4 induced higher proportions of treated with either RA or Pam_3CSK_4 alone (Fig. 7C, D). Moreover, TLR1/2 stimulation and RA synergized to confer Spleen-DC with the capacity to induce IgA-ASC upon B cell activation (Fig. 7E), an effect that correlated with the upregulation of *April*, *Baff*, and *Nos2* in DC (encoding APRIL, BAFF and iNOS, respectively) (Fig. S6), all of which are important factors for inducing IgA-ASC [25]. Of note, RA-treated human Mo-DC did not enhance IgA production by polyclonally activated human B cells (data not shown), suggesting that Mo-DC (differentiated with IL-4 and GM-CSF) are not permissive for IgA class-switching or that

there are some species-specific differences in this regard. Therefore, in addition to modulating RA synthesis and gut-homing imprinting, RA- and MyD88-dependent pathways synergize to confer DC with the capacity to induce Foxp3 T cells and IgA-ASC, which are hallmarks of mucosal-specific imprinting.

Discussion

RA is synthesized from all-*trans* retinol, which is obtained from the food as all-*trans* retinol, retinyl esters or β-carotenes (provitamin A) [26]. Interestingly, we found that retinoids follow a proximal-to-distal gradient in the intestine, with the highest levels being found in the duodenum and jejunum. This retinoid gradient might be explained, at least in part, by differences in the absorption and/or synthesis of retinoids along the intestinal tract, which is supported by the higher levels of carotenoid receptor SI-BI (scavenger receptor type B, class I) and carotenoid metabolizing enzyme BCMO1 (β,β-carotene-15,15′-monooxygenase 1) found in the proximal intestine as compared to ileum [27]. A similar proximal-to-distal expression gradient has been described for cellular retinol binding protein (CRBP)-II and LRAT [28], which might contribute to further enhance the pool of retinol and retinyl esters found in the proximal intestine (see also Supp. Text-2).

A model in which RA is physiologically required to induce its own synthesis implies the existence of another (primary) source of RA to educate DC. In this regard, it has been shown that intestinal epithelial cells (IEC) express high levels of Raldh1 [30, 31] and can produce RA [7, 32]. In fact, in contrast to Raldh2 which is decreased in MLN-DC from VAD mice (our data and ref. [17]), Raldh1 expression in IEC does not require RA [32, 33], suggesting that IEC might be either “hard-wired” to synthesize RA or that their RA-synthesizing capacity depends on other environmental factors (e.g., TLR signals, microbiota). Thus, IEC might provide, at least in part, a primary supply of RA in the gut mucosa, which in turn induces Raldh2 in gut-associated DC, conferring them with RA-synthesizing capacity (see also Supp. Text-2).

A recent report suggested that GM-CSF is physiologically required for gut-associated DC education and that RA might be important for DC education by inducing GM-CSF-expressing macrophages in MLN [17]. However, we did not observe impairment in *Aldh1a2* mRNA expression, Raldh activity or gut-homing imprinting capacity in gut-associated DC from GM-CSF$^{-/-}$ mice (data not shown), suggesting that GM-CSF may not be essential for gut-associated DC education *in vivo*. These discrepancies could be partially explained by the fact that we used DC from mice deficient only in GM-CSF, whereas Yokota et al. isolated DC from mice deficient in the common β-c subunit, which is shared by GM-CSF, IL-3 and IL-5 receptors [17].

Similar to TLR2 stimulation [20], RA-mediated DC education also required signaling via ERK/MAPK. This finding is in line with previous studies showing that some RA-mediated effects depend on ERK/MAPK, but not on p38/MAPK or JNK/MAPK signaling [35]. However, despite the fact that RA share some signaling mechanisms with TLR2 stimulation, including ERK/MAPK and MyD88, these pathways are not redundant, because we observed additive and even synergistic effects on DC education when pre-treating DC with RA and a TLR1/2-agonist (see also Supp. Text-3).

We show that RA is a critical factor in gut-associated DC education, which is necessary and sufficient to confer DC with key gut-specific imprinting properties, including the capacity to synthesize RA, imprint gut-tropic lymphocytes and T_{REG}, as well as promote IgA-ASC differentiation (Fig. S7). Nevertheless, some of our results in murine DC were not

completely reproduced using human Mo-DC and further investigation will be needed to determine whether there are some species-specific differences in this regard.

In conclusion, our data highlight an unexpected link between RAR nuclear receptor and MyD88/TLR-dependent pathways, indicating that gut-associated DC specialization integrates signals derived from both dietary components acting on nuclear receptors, and from the intestinal microbiota acting via pathogen-recognition receptors. These signals could be eventually manipulated to improve vaccination strategies aimed at enhancing intestinal immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: EJV was supported by grants from Crohn's & Colitis Foundation of America (CCFA). MRB and MR were supported by Fondecyt Grants 1100557 and 1100448 and PFB16 from Conicyt. UHvA was supported by grants from NIH. JRM was supported by grants from Crohn's & Colitis Foundation of America (CCFA), Cancer Research Institute (CRI), Howard H. Goodman (MGH), Massachusetts Life Science Center (MLSC) and NIH DP2 2009A054301.

We thank Drs. Barbara Cassani, Oscar Pello and Scott Snapper for comments on this manuscript. JRM is indebted to Ingrid Ramos for constant support.

References

1. Mora JR. Homing imprinting and immunomodulation in the gut: role of dendritic cells and retinoids. Inflamm Bowel Dis. 2008; 14:275–89. [PubMed: 17924560]
2. Eksteen B, Adams DH. GSK-1605786, a selective small-molecule antagonist of the CCR9 chemokine receptor for the treatment of Crohn's disease. IDrugs. 2010; 13:472–781. [PubMed: 20582872]
3. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Rosemblatt M, von Andrian UH. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature. 2003; 424:88–93. [PubMed: 12840763]
4. Johansson-Lindbom B, Svensson M, Wurbel MA, Malissen B, Marquez G, Agace W. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. J Exp Med. 2003; 198:963–969. [PubMed: 12963696]
5. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. Science. 2006; 314:1157–60. [PubMed: 17110582]
6. Eksteen B, Mora JR, Haughton EL, Henderson NC, Turner LL, Villablanca EJ, Curbishley SM, Aspinall AI, von Andrian UH, Adams DH. Gut Homing Receptors on CD8 T Cells Are Retinoic Acid Dependent and Not Maintained by Liver Dendritic or Stellate Cells. Gastroenterology. 2009; 137:320–329. [PubMed: 19233184]
7. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004; 21:527–38. [PubMed: 15485630]
8. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science. 2007; 317:256–60. [PubMed: 17569825]
9. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med. 2007; 204:1775–1785. [PubMed: 17620362]
10. Szatmari I, Pap A, Ruhl R, Ma JX, Illarionov PA, Besra GS, Rajnavolgyi E, Dezso B, Nagy L. PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. J Exp Med. 2006; 203:2351–62. [PubMed: 16982809]

11. Kawai T, Akira S. TLR signaling. Semin Immunol. 2007; 19:24–32. [PubMed: 17275323]
12. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998; 9:143–50. [PubMed: 9697844]
13. Svensson M, Johansson-Lindbom BFZ, Jaenssona E, Austenaa LM, Blomhoff R, Agace WW. Retinoic acid receptor signaling levels and antigen dose regulate gut homing receptor expression on CD8+ T cells. Mucosal Immunology. 2008; 1:38–48. [PubMed: 19079159]
14. O'Byrne SM, Wongsiriroj N, Libien J, Vogel S, Goldberg IJ, Baehr W, Palczewski K, Blaner WS. Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT). J Biol Chem. 2005; 280:35647–57. [PubMed: 16115871]
15. Lee SH, Hu LL, Gonzalez-Navajas J, Seo GS, Shen C, Brick J, Herdman S, Varki N, Corr M, Lee J, Raz E. ERK activation drives intestinal tumorigenesis in Apc(min/+) mice. Nat Med. 2010; 16:665–70. [PubMed: 20473309]
16. Kane MA, Napoli JL. Quantification of endogenous retinoids. Methods Mol Biol. 2010; 652:1–54. [PubMed: 20552420]
17. Yokota A, Takeuchi H, Maeda N, Ohoka Y, Kato C, Song SY, Iwata M. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. Int Immunol. 2009; 21:361–77. [PubMed: 19190084]
18. Saurer L, McCullough KC, Summerfield A. In vitro induction of mucosa-type dendritic cells by all-trans retinoic Acid. J Immunol. 2007; 179:3504–14. [PubMed: 17785784]
19. Sun D, Ding A. MyD88-mediated stabilization of interferon-gamma-induced cytokine and chemokine mRNA. Nature immunology. 2006; 7:375–81. [PubMed: 16491077]
20. Manicassamy S, Ravindran R, Deng J, Oluoch H, Denning TL, Kasturi SP, Rosenthal KM, Evavold BD, Pulendran B. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. Nat Med. 2009; 15:401–9. [PubMed: 19252500]
21. Moore WT Jr, Murtaugh MP, Davies PJ. Retinoic acid-induced expression of tissue transglutaminase in mouse peritoneal macrophages. J Biol Chem. 1984; 259:12794–802. [PubMed: 6149218]
22. Schug TT, Berry DC, Shaw NS, Travis SN, Noy N. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. Cell. 2007; 129:723–33. [PubMed: 17512406]
23. Canon E, Cosgaya JM, Scsucova S, Aranda A. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. Mol Biol Cell. 2004; 15:5583–92. [PubMed: 15371543]
24. Li Y, Hashimoto Y, Agadir A, Kagechika H, Zhang X. Identification of a novel class of retinoic acid receptor beta-selective retinoid antagonists and their inhibitory effects on AP-1 activity and retinoic acid-induced apoptosis in human breast cancer cells. J Biol Chem. 1999; 274:15360–6. [PubMed: 10336422]
25. Tezuka H, Abe Y, Iwata M, Takeuchi H, Ishikawa H, Matsushita M, Shiohara T, Akira S, Ohteki T. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature. 2007; 448:929–33. [PubMed: 17713535]
26. Mora JR, Iwata M, von Andrian UH. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol. 2008
27. von Lintig J. Colors With Functions: Elucidating the Biochemical and Molecular Basis of Carotenoid Metabolism. Annu Rev Nutr. 2010; 30:35–56. [PubMed: 20415581]
28. Herr FM, Wardlaw SA, Kakkad B, Albrecht A, Quick TC, Ong DE. Intestinal vitamin A metabolism: coordinate distribution of enzymes and CRBP(II). J Lipid Res. 1993; 34:1545–54. [PubMed: 8228637]
29. Stenstad H, Svensson M, Cucak H, Kotarsky K, Agace WW. Differential homing mechanisms regulate regionalized effector CD8alphabeta+ T cell accumulation within the small intestine. Proc Natl Acad Sci U S A. 2007; 104:10122–7. [PubMed: 17551016]
30. Lampen A, Meyer S, Arnhold T, Nau H. Metabolism of vitamin A and its active metabolite all-trans-retinoic acid in small intestinal enterocytes. J Pharmacol Exp Ther. 2000; 295:979–85. [PubMed: 11082432]

31. Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal Immunol. 2009

32. Frota-Ruchon A, Marcinkiewicz M, Bhat PV. Localization of retinal dehydrogenase type 1 in the stomach and intestine. Cell Tissue Res. 2000; 302:397–400. [PubMed: 11151452]

33. Bhat PV. Retinal dehydrogenase gene expression in stomach and small intestine of rats during postnatal development and in vitamin A deficiency. FEBS Lett. 1998; 426:260–2. [PubMed: 9599020]

34. Edele F, Molenaar R, Gutle D, Dudda JC, Jakob T, Homey B, Mebius R, Hornef M, Martin SF. Cutting edge: instructive role of peripheral tissue cells in the imprinting of T cell homing receptor patterns. J Immunol. 2008; 181:3745–9. [PubMed: 18768825]

35. Battle TE, Roberson MS, Zhang T, Varvayanis S, Yen A. Retinoic acid-induced blr1 expression requires RARalpha, RXR, and MAPK activation and uses ERK2 but not JNK/SAPK to accelerate cell differentiation. Eur J Cell Biol. 2001; 80:59–67. [PubMed: 11211936]

36. Suzuki K, Maruya M, Kawamoto S, Sitnik K, Kitamura H, Agace WW, Fagarasan S. The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut. Immunity. 2010; 33:71–83. [PubMed: 20643338]

37. Zou F, Liu Y, Liu L, Wu K, Wei W, Zhu Y, Wu J. Retinoic acid activates human inducible nitric oxide synthase gene through binding of RARalpha/RXRalpha heterodimer to a novel retinoic acid response element in the promoter. Biochem Biophys Res Commun. 2007; 355:494–500. [PubMed: 17306764]

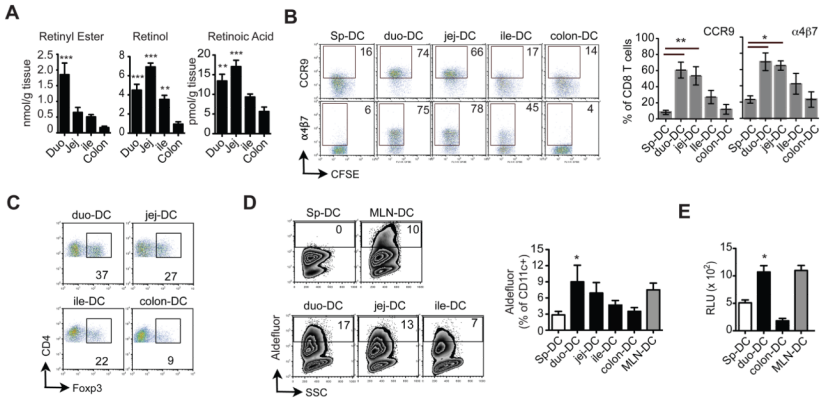

Figure 1. DC ability to imprint gut homing correlates with retinoid levels in the gut

(**A**) Quantification of retinyl esters, retinol and RA in duodenum (duo), jejunum (jej), ileum (ile) and colon (*n*=6). (**B**) LP-DC or PP-DC from duo, jej, ile, colon were used to activate naïve CD8 T cells. After 4–5 days T cells were analyzed for α4β7 and CCR9 expression (*n*=5). (**C**) DC were used to activate naïve CD4 T cells. After 4 days T cells were analyzed for Foxp3 expression. FACS plots are representative of two independent experiments. (**D**) Raldh activity in DC (*n*=4). (**E**) Luciferase activity in DC from DR5-luciferase mice (*n*=4). Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001

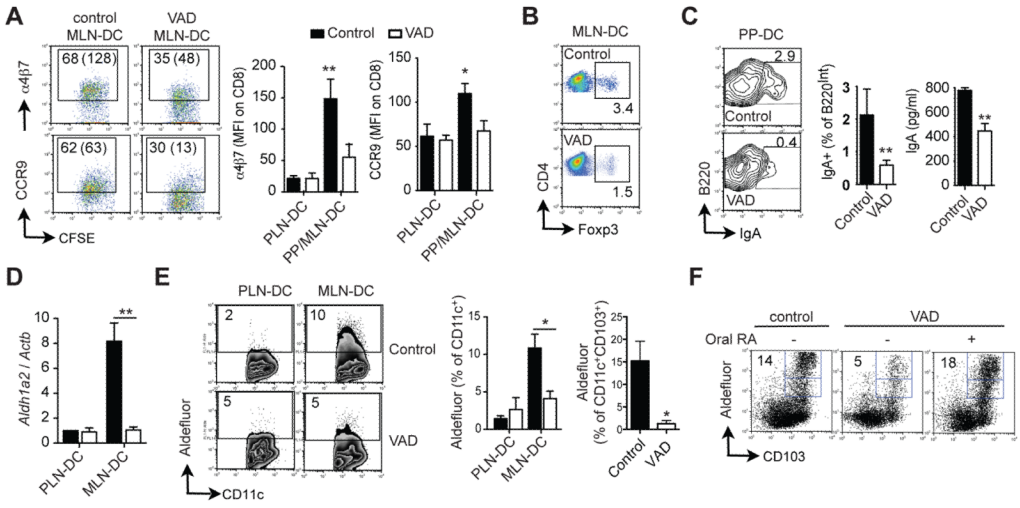

Figure 2. RA is necessary in vivo for gut-associated DC education

(**A–G**) DC were isolated from mice on a vitamin A deficient (VAD) or control diet. (**A**) DC were used to activate naïve CD8 T cells. After 4–5 days T cells were analyzed for α4β7 and CCR9 expression (*n*=4). (**B**) DC were used to activate naïve CD4 T cells. After 4–5 days T cells were analyzed for Foxp3 expression. FACS plots are representative of two independent experiments. (**C**) DC were co-cultured with naïve B cells activated with anti-IgM plus IL-5. After 4 days the co-cultures were analyzed for intracellular IgA in $B220^{Int}$ cells and IgA in the supernatant (*n*=4). (**D**) *Aldh1a2* mRNA and (**E**) Raldh activity in $CD11c^+$ and $CD11c^+CD103^+$ DC (*n*=5). (**F**) Raldh activity in $CD11c^+$ DC from control or VAD mice ± oral RA supplementation. FACS plots are representative of two independent experiments. Mean ± SEM, *p<0.05, **p<0.01

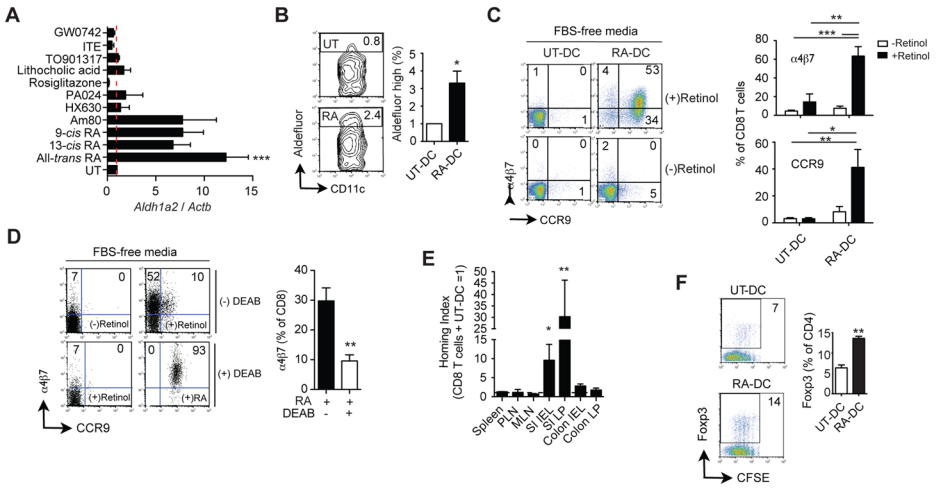

Figure 3. RA is sufficient to confer DC with RA synthesizing capacity in vitro

(**A**) *Aldh1a2* mRNA in Spleen-DC incubated for 24 h with 100 nM all-*trans* RA (RA) or 100 nM of the indicated nuclear receptor agonists. HX630 and PA024 were used at 1 or 10 μM, with similar results (*n*=5–10). (**B–F**) Spleen-DC were incubated for 24 h with or without 100 nM RA (RA-DC and UT-DC, respectively) and analyzed for (**B**) Raldh activity (*n*=5) or (**C**) washed, pulsed with antigen and used to activate naïve CD8 T cells from TCR transgenic OT-1xRAG2$^{-/-}$ mice in FBS-free media ± 50 nM retinol. After 4–5 days T cells were analyzed for α4β7 and CCR9 expression (*n*=7). (**D**) Co-cultures were performed as described in (**C**) either in the presence or absence of the Raldh inhibitor DEAB (*n*=7). (**E**) Competitive homing experiment between CD8 T cells activated with RA-DC or UT-DC (*n*=5). (**F**) Naïve CD4 T cells from OT-1xRAG2$^{-/-}$ mice were activated with UT-DC or RA-DC. Five days later CD4 T cells were analyzed for Foxp3 expression (*n*=3).

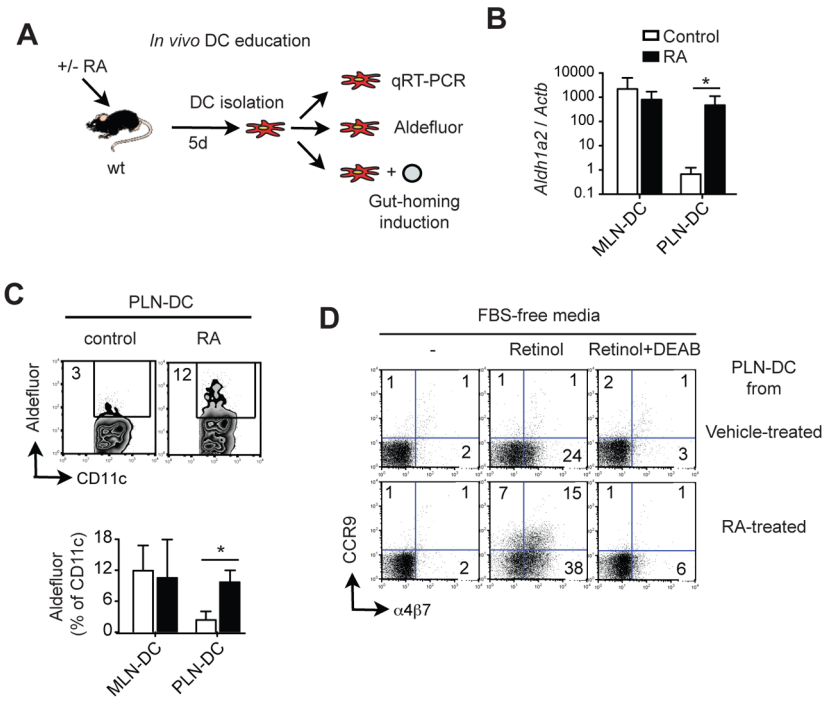

Figure 4. RA is sufficient to confer DC with RA synthesizing capacity in vivo

(**A**) Wild type mice were supplemented orally with RA (400 μg/dose) every other day for 6 days. After that, MLN-DC and PLN-DC were isolated and analyzed for the expression of (**B**) *Aldh1a2* mRNA and (**C**) Raldh activity (n=5). (**D**) PLN-DC from control or RA-treated mice were used to activate naïve CD8 T cells in FBS-free media ± retinol and either in the presence or absence of DEAB. FACS plots are representative of two independent experiments. Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001

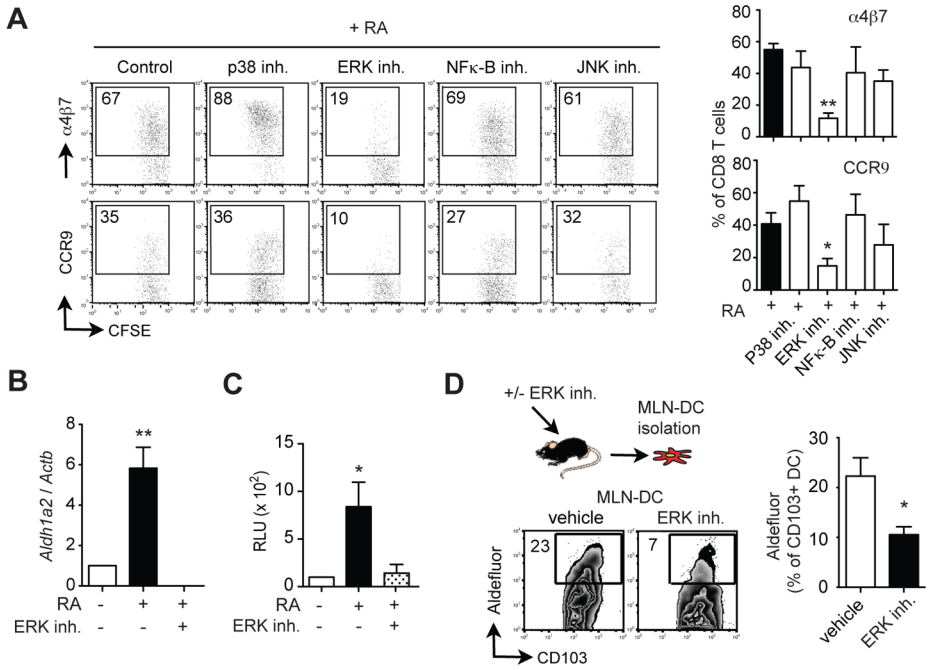

Figure 5. DC education requires ERK/MAPK signaling

(**A–C**) Spleen-DC were incubated for 24 h with or without 100 nM RA and in the presence or the absence of inhibitors for p38 (SB203580, 10 μM), ERK (U0126, 10 μM), NF-κB (SN50, 50 μM) or JNK (SP600125, 50 μM). (**A**) DC were used to activate naïve CD8 T cells. After 4–5 days T cells were analyzed for α4β7 and CCR9 expression (*n*=9). (**B**) *Aldh1a2* mRNA expression in DC (*n*=3). (**C**) Spleen-DC from DR5-luciferase mice were incubated for 24 h with or without RA and in the presence or absence of the ERK inhibitor (U0126, 10 μM) and analyzed for their luciferase activity (*n*=3). (**D**) Wild type mice were orally treated with the ERK inhibitor PD0325901 (25 μg/g/dose) every other day for 6 days. After that, $CD11c^+$ MLN-DC were analyzed for CD103 expression and Raldh activity (*n*=5). Mean ± SEM, *p<0.05, **p<0.01

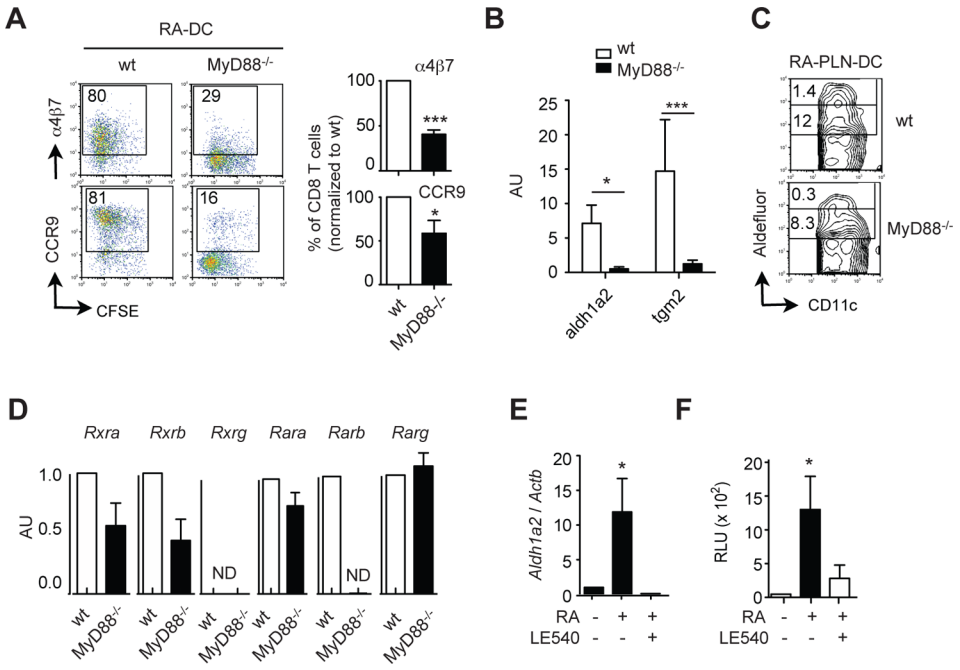

Figure 6. MyD88 is required for RA-mediated DC education

(**A**–**B**) Wild type or MyD88$^{-/-}$ Spleen-DC were incubated with 100 nM RA for 24 h. (**A**) DC were used to activate naïve CD8 T cells. After 4–5 days T cells were analyzed for α4β7 and CCR9 expression (*n*=5). (**B**) *Aldh1a2* (*n*=4) or *Tgm2* (*n*=5) mRNA expression in DC. (**C**) Wild type or MyD88$^{-/-}$ mice were supplemented orally with RA (400 μg/dose) every other day for 6 days and PLN-DC were analyzed for Raldh activity (*n*=7). (**D**) Spleen-DC from wild type or MyD88$^{-/-}$ mice were analyzed for their expression of *Rxra, Rxrb. Rxrg, Rara, Rarb* and *Rarg* mRNA (*n*=4–6). ND: not detected. (**E**) Spleen-DC were incubated for 24 h with or without 100 nM RA and either in the presence or absence of the RAR® inhibitor LE540 (1 μM) and then analyzed for *Aldh1a2* mRNA expression (*n*=3). (**F**) Spleen-DC from DR5-luciferase mice were incubated for 24 h with or without 100 nM RA and either in the presence or absence of LE540 and then analyzed for their luciferase activity (*n*=3). Mean ± SEM, *p<0.05, ***p<0.001

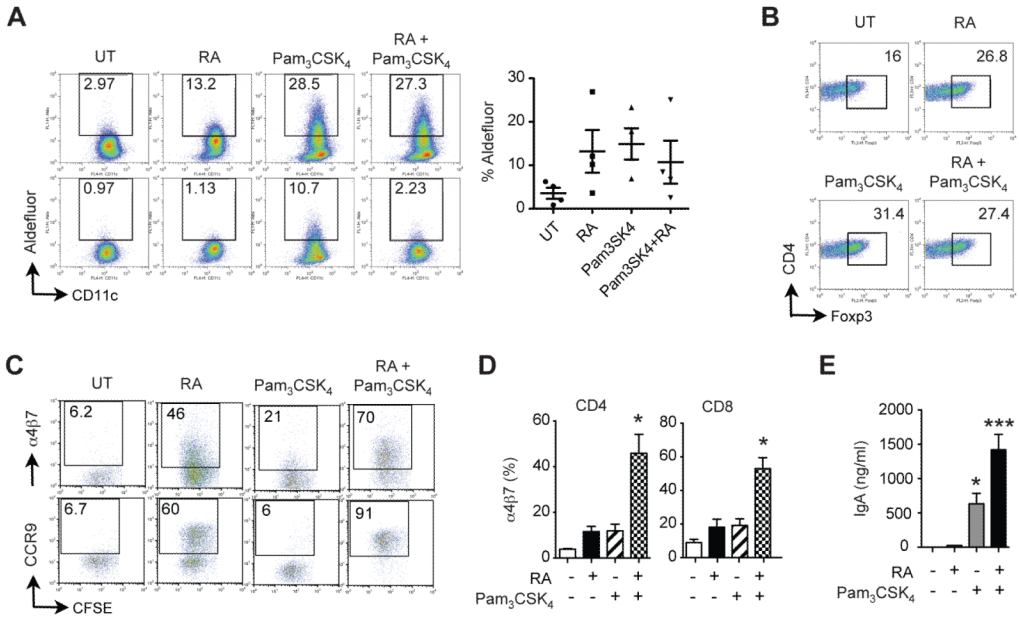

Figure 7. TLR signals contribute to RA-mediated human and murine DC education

(**A**) Monocyte-derived DC (Mo-DC) from healthy donors were treated with either RA (100 nM), Pam_3CSK_4 (0.5 μg/ml) or both from day 3 of differentiation. Raldh activity was analyzed at day 6 in $CD11c^+$ cells (*n*=4). FACS plots show representative results in Mo-DC from two donors. (**B**) Mo-DC pretreated with either RA, Pam_3CSK_4 or both were washed and cultured with allogenic CD4 T cells activated with anti-CD3 plus anti-CD28 and hTGF-β1 (2 ng/ml). After 4 days, CD4 T cells were analyzed for Foxp3 expression. FACS plots show one representative experiment out of two. (**C**) Spleen-DC were incubated for 24 h in the presence of 100 nM of RA, Pam_3CSK_4 (0.5 μg/ml) or both, washed and used to activate naive CD8 T cells. After 4 days, T cells were analyzed for their expression of α4β7 and CCR9. FACS plots are representative of three experiments. (**D**) Human Mo-DC were treated at day 6 with or without 100 nM of RA, Pam_3CSK_4 (0.5 μg/ml) or both for 24 h, washed and co-cultured with total human T cells activated with plate-bound anti-CD3 plus anti-CD28 antibodies. After 6 days, CD4 and CD8 T cells were analyzed for their expression of α4β7 (*n*=3). (**E**) Spleen-DC were incubated for 24 h in the presence of 100 nM of RA, Pam_3CSK_4 (0.5 μg/ml) or both, washed and co-cultured with naïve B cells activated with anti-IgM plus IL-5. After 4 days IgA levels were measured in the culture supernatants (*n*=7). Mean ± SEM, *p<0.05, ***p<0.001